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Enzymatic and Molecular Biochemical Characterizations of Human Neutrophil Elastases and a Cathepsin G-like Enzyme

Woo Mi Kim* and Kooil Kang

Department of Pharmacology, Kosin University Medical College, Pusan 602-702, Korea.

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Human neutrophil elastase (HNE, EC 3. 4. 21. 37) is a causative factor of inflammatory diseases, including emphysema and rheumatoid arthritis. Enzymatic characterization is important for the development of new drugs involved in the regulation of this enzyme. In this study, we investigated the enzymatic and biochemical properties of five different elastolytic enzymes, with a molecular mass between 24 kDa and 72 kDa. Three elastases, molecular masses of 27, 29, 31 kDa, might be elastase isozymes that have the same NH_2-terminal amino acid sequences of Ile-Val-Gly-Gly-Arg-Arg-Ala. The 24-kDa enzyme, which showed the identical NH_2-terminal amino acid sequences to elastase, was a degraded fragment of native elastase. The elastolytic activity was conserved at the 6/7 domain of the NH_2-terminal region. The inhibitory characteristics of PMSF, DipF were the same as those of native elastases. The 72-kDa molecule, which showed elastolytic activity, might be a trimer formed between native elastases (31 kDa and 29 kDa) and a cathepsin G-like enzyme, which did not show elastolytic activity but enhanced the elastolytic activity of neutrophil elastase. Although this cathepsin G-like enzyme showed weak cathepsin G activity, it has distinguishable NH_2-terminal sequences of Ile-Val-Gly-Gly-Ser-Arg-Ala- from those of elastase or cathepsin G. The potentiation of elastolytic activity could be a result of the trimerization of native elastase with a cathepsin G-like enzyme, and was then weakly inhibited by serine protease inhibitors, such as PMSF, DipF. Therefore, we suggest the cathepsin G-like enzyme to be a novel enzyme, which has an important role in the development of inflammation.

Keywords: Cathepsin G-like Enzyme; Human Neutrophil Elastase; Isozymes.

Introduction

Human neutrophil elastase (HNE, EC 3.4.21.37) is one of the serine proteases, which plays an important role in the degradation of host connective tissues in pulmonary emphysema (Mittan, 1972) and rheumatoid arthritis (Glynn, 1972; Starky *et al.*, 1977). Enzymatic characterization of elastase is essential for the development of specific inhibitors to HNE, and purified elastases are required to investigate the biochemical properties of elastases. However, previous studies showed discrepancies in the molecular weight of HNE. Ohlsson and Olsson (1974) have described the leukocyte elastases with molecular weights of 32–36,000. In contrast to their observation, Taylor has described the purification of HNElastase with a molecular mass of 22,000 (Taylor and Crawford, 1975). Schmidt and Havemann using leukocyte granules obtained a molecular weight for HNE of 27,000 by gel filtration (Schmidt and Havemann, 1977). These variant descriptions about the molecular weights of HNElastases might be an impediment to the consistant analysis of the enzyme. In our previous study, purified elastase showed five different molecular weight with bands between 72,000 and 24,000 in SDS-PAGE, and the electrophoretic mobility differences of the these elastases remains unexplained. Furthermore, separation of HNElastases with different molecular weights is very difficult since their molecular weights are similar and their biochemical properties are unstable. Therefore, this paper describes a modified method for the purification of HNElastases using multiple chromatographic steps and subsequent characterization by N-terminal amino acid sequence analysis.

* To whom correspondence should be addressed.
Tel: 82-51-240-6437; Fax: 82-51-241-5458
E-mail: kwm@ns.kosinmed.or.kr

Abbreviations: DIPF, diisopropylfluorophosphate; HNE, human neutrophil elastase; PMSF, phenylmethylsulfonylfluoride.

Materials and Methods

Materials *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-Nitroanilide (SAPNA), *N*-Succinyl-Ala-Ala-Ala- *p*-Nitroanilide (SANA), sodium acetate trihydrate, trifluoroacetic acid (TFA), hydrochloric acid (HCl), 4-vinyl pyridine, guanidine hydrochloride, phenylmethylsulfonyl fluoride (PMSF), diisopropylfluorophosphate (DipF), calcium chloride, and phenylisothiocyanate (PITC) were obtained from the Sigma Chemical Co., St. Louis, USA. Acetonitrile, methanol, and acetic acid were of HPLC grade and were obtained from Fisher Scientific Co., Fair Lawn, NJ. Pyridine, triethylamine (TEA), heptane, ethyl acetate, and *n*-butyl acetate (sequencing grade) were obtained from Aldrich. Tris, glycine, sodium dodecyl sulfate, 2-mercaptoethanol, and dithiothreitol (DTT) were obtained from Bio-Rad, Richmond, USA. Spectrapor dialysis membranes were from Spectrum medical industries, Inc., LA, USA, ultrafiltration membranes were obtained from Amicon Co., Denvers, MA, USA. The Pico-Tag™ column, TSK-250 gel filtration column, and μ BONDAPAK™ C18 reverse phase column were purchased from Waters Co. Human whole blood was supplied by the Blood Bank of Kosin Medical Center. All other reagents were of the highest available grade.

Purification of human neutrophil elastases

Preparation of leukocyte extracts Leukocytes were isolated from whole blood by the modified method of Baugh *et al*. (1976) and Kang *et al*. (1985). In brief, human neutrophils were separated from whole blood by ficoll-hypaque step-gradient centrifugation at $200 \times g$ for 20 min and washed three times with 50 mM Tris-Cl buffer, pH 7.3. Separated neutrophils were suspended in 0.5 M Tris-Cl buffer, pH 7.3, containing 1.5 M NaCl and 0.05 M $CaCl_2$, homogenized with Polytron and centrifuged at $30{,}000 \times g$ for 60 min (Jeung *et al*., 1987). The supernatant was then collected.

Ultrogel AcA 54 gel filtration chromatography Leukocyte extract was chromatographed through an AcA 54 gel filtration column equilibrated with 50 mM of Tris-Cl buffer containing 150 mM NaCl. Elastase-rich fractions were pooled, concentrated, and dialyzed against 50 mM sodium acetate (NaOAc), pH 4.5, containing 150 mM NaCl.

CM-Sephadex 25 ion exchange chromatography Elastase, purified by ultrogel AcA 54 chromatography, was chromatographed again with a CM-Sephadex 25 ion exchange column. The column was equilibrated with 50 mM NaOAc buffer, pH 4.5, containing 150 mM of NaCl, and bound protein was eluted with a linear salt gradient of 0.15–0.7 M NaCl in the same buffer.

High performance liquid chromatography by a Biosil TSK-250 gel filtration column Two hundred microliters of the concentrated elastase obtained from the previous separation was applied to a Biosil TSK-250 column equilibrated in 50 mM Tris-Cl, pH 7.3, containing 0.15–0.5 M NaCl and 2 mM DTT. The flow rate of eluent was 1 ml/min. The protein profile was monitered continuously at a wavelength of 280 nm.

Polyacrylamide gel electrophresis SDS-PAGE was performed using a modification of the method of Laemmli (1970).

Quantitation of protein concentrations The protein concentration of purified enzyme was determined by the method of Lowry *et al*. (1951).

Enzyme assay

Elastase assay with synthetic substrate, N-Succinyl-Ala-Ala-Ala-p-Nitroanilide (SANA) Ten microliters of enzyme was added to 50 mM Tris-Cl, pH 7.3, containing 150 mM NaCl, 5 mM $CaCl_2$, 0.1% Brij 35, and then SANA was added to the reaction medium and the final concentration was 0.6–3 mM, depending upon the design of the experiment (Kang, 1985). The reaction was carried out at 37°C for 10–60 min and the concentration of liberated *p*-nitroaniline was monitered by absorbance at 410 nm using a Titertek Multiskan Spectrophotometer (MCC/340, Flow laboratories, Switzerland). Enzyme units were defined as nmole of nitroaniline released per hour, assuming a molar extinction coefficient of 8,800 M^{-1} cm^{-1}.

Elastase assay with insoluble elastin Elastase activity was measured using elastin-Congo red as a substrate (Shotton *et al*., 1970). Briefly, 0.5 ml of enzyme was incubated with 10 mg of elastin-Congo red in 50 mM Tris-Cl, pH 7.3, containing 150 mM NaCl at 37°C for 18–24 h. The reaction was stopped by centrifugation at $30{,}000 \times g$ for 2 min and the absorbance was read at 495 nm. One unit of enzyme was defined as the amount producing a 1,000 absorbance deviation at 495 nm per min per ml.

Inhibition of elastase activity PMSF and DipF were tested at concentrations ranging from 20 μM to 1 mM. Inhibition experiments were carried out by mixing 10 μl of purified elastase, with varying amounts of the different inhibitors in 200–300 μl of reaction medium containing 50 mM Tris-Cl, pH 7.3. The mixtures were pre-incubated for 20 min at 37°C, and then the substrates, SANA or insoluble elastin, were added to the reaction medium and incubated for 0.5–24 h at 37°C. Liberated *p*-nitroaniline or soluble elastin were measured as described. The elastase activity in the presence of the inhibitor was expressed as a percentage compared with the control.

Analysis of the NH_2-terminal amino acid sequence

Pyridylethylation of elastase Purified elastase was reduced in 200 μl of 6 M guanidium chloride, 0.1 M Tris-Cl, pH 8.5, containing 0.1% 2-mercaptoethanol at 37°C for 30 min and alkylated by the addition of 4-vinylpyridine to a final concentration of 1% at 18°C for 3 h in the dark. The S-pyridylethylated elastase was precipitated by the addition of 4 volumes of methanol-acetone (v/v = 50/50) solution and kept at −20°C for overnight. The precipitate was collected by centrifugation at $10{,}000 \times g$ for 30 min and was diluted with 0.1% trifluoroacetic acid. Diluted elastase was purified by HPLC with a μ BONDAPAK™ C18 reverse-phase column. Bound protein was eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid.

Sequence analysis of S-pyridylethylated elastase Purified elastase was dissolved in 50% pyridine (200 μl), and sequential Edman degradation was carried out. Phenylthiohydantoin derivatives were identified by HPLC using a Pico-Tag™ column eluted with a gradient of 5–65% acetonitrile in 1.4 mM sodium acetate, pH 5.4, containing 0.1% triethylamine at 38°C.

Results

The leukocyte extract was followed by sequential gel filtration and ion exchange chromatography (Figs. 1A and 1B). The CM-sephadex 25 ion exchange chromatography (IEC) was crucial for separating elastase fractions from the fractions which demonstrated cathepsin G activity. The elastase fractions which purified from ion exchange chromatography were pooled and chromatographed by HPLC using a TSK-250 gel filtration column. There were 4 major protein peaks (Fig. 2). The molecular mass of peak b was 72 kDa. Peak c migrated as three discrete bands with apparent molecular masses of 31, 29, and 27 kDa in SDS-PAGE. Peak d consists of a single polypeptide band with a molecular mass near 24 kDa.

To investigate the quantity of fraction d that might be related to the effect of the autodigestion of fraction c, the fraction c of Fig. 2 was rechromatographed immediatly or 24 h later (Fig. 3). The total amount of the protein of fraction d was increased dependently by time (Fig. 3B). Thus fraction d, the autolytic cleavage product, was taken to represent the effect of autolysis on enzyme activity (Fig. 4B).

The fraction c in Fig. 2 was further purified separately by HPLC using a TSK-250 gel filtration column under 0.5 M NaCl and we obtained four peaks, represented as c1, 2, 3, 4 in Fig. 4A.

To investigate the enzymatic property of each peak, we compared the elastase activity on elastin degradation or SANA hydrolysis and the inhibitory effects of PMSF, DipF. Three elastases, corresponding to c1, 2, 3, respectively, showed a similar capability of digesting elastin or SANA (Table 1). But fraction c4 had no elastolytic activity (Table 1). The fraction d showed a similar activity on SANA or elastin to the native elastase (Table 1). Those activities were effectively inhibited by 1 mM PMSF and 20 μM DipF (Tables 2 and 3). On the other hand, fraction b has marked elastolytic activity on elastin degradation but not on SANA hydrolysis (Table 1).

To determine whether each peak with a different molecular weight is, or is not, an identical enzyme, we analyzed the NH_2-terminal amino acid sequences of c1-4, d and compared them to those of HNElastase (Table 4). The NH_2-terminal amino acid sequences of c1, 2, 3 and d were same as Ile-Val-Gly-Gly-Arg-Arg-Ala. The 72 kDa molecule (fraction b) was reduced and

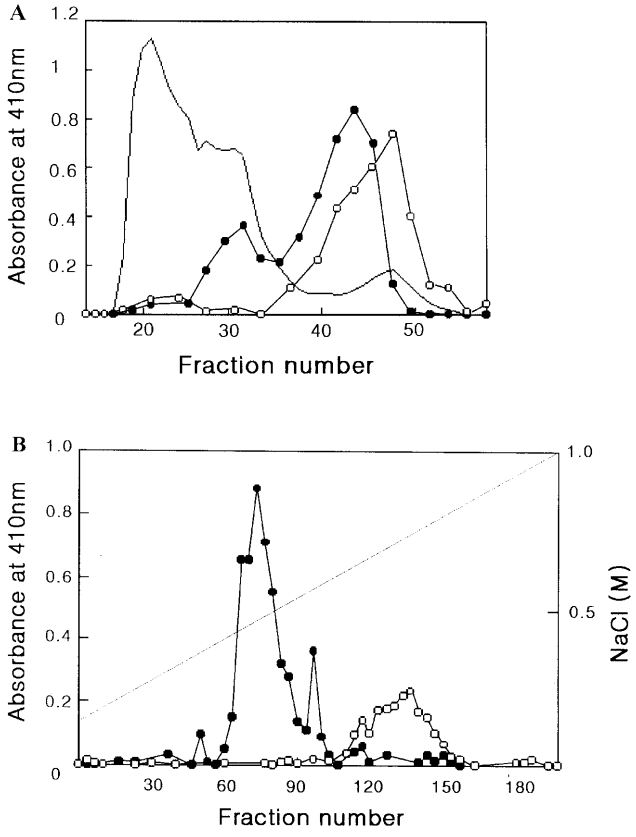

Fig. 1. Purification of human neutrophil elastase by two sequential chromatography. **A.** Chromatography of leukocyte extract by a Ultrogel AcA 54 gel filtration column. The column (2.5 × 100 cm) was equilibrated with 50 mM Tris-Cl, pH 7.3, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.1% Brij 35 at 4°C. Ten milliliters of leukocyte extract was loaded and eluted at flow rate of 1 drop/6–7 s and 8 ml of each fraction was collected. The distribution of protein absorbance at 280 nm as —, elastase activity against SANA as –●–, cathepsin G activity against SAPNA as –○–, are shown. The fractions 38–46 containing elastase and cathepsin G activity were pooled for further purification. **B.** Chromatography of partially purified elastase by a CM-Sephadex 25 ion exchange column. The column (1.5 × 18 cm) was eluted with a linear gradient of 0.15–1 M NaCl (—) in 50 mM NaOAc, pH 5.5, containing 0.1% Brij 35 at 4°C, and 2.5 ml of eluent was collected in each fraction at a flow rate of 1 drop/16 s. The distribution of elastase and cathepsin G activity were shown as –●– and –○–, respectively.

rechromatographed by HPLC using a TSK-250 gel filtration column. Then we obtained three peaks, c1, 2 and 4 (Fig. 5). These results showed that the 72-kDa molecule might be a trimer formed between the fractions c1, 2 and 4. In contrast to the enzymatic properties of fraction c1-3, fraction c4 did not show elastolytic activity with SANA and elastin. However it stimulated the elastoytic activity of native elastase, c1, 2, since the 72 kDa molecule, suggesting a trimer, showed enhanced activity on elastin degradation compared to that of native elastase (Table 1). This elastolytic activity

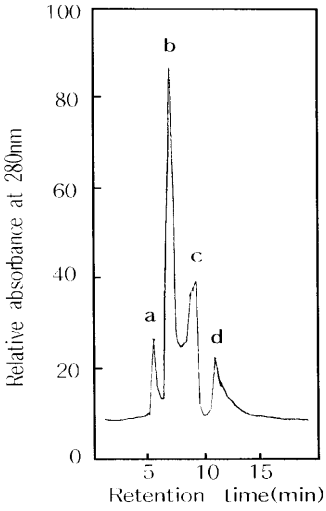

Fig. 2. TSK-250 gel filtration column chromatography of purified elastase from IEC. Concentrated elastase from the previous purification by IEC in Fig. 1B was dialyzed against 50 mM Tris-Cl, pH 7.3, containing 0.15 M NaCl and 0.1% Brij, and loaded onto the TSK-250 gel filtration column and eluted at a flow rate of 1 ml/min. The protein profile was monitored continuously at a wavelength of 280 nm.

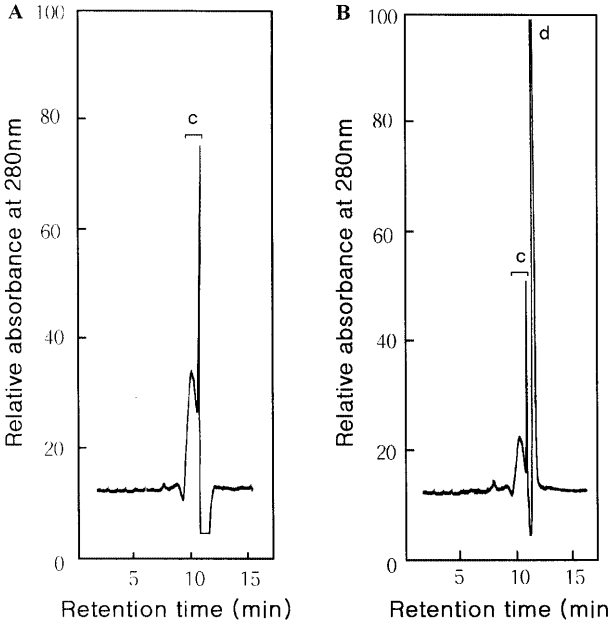

Fig. 3. TSK-250 gel filtration column chromatography of fraction c in Fig. 2. Fraction c, which was eluted at 9–11 min in Fig. 2, was rechromatographed immediately (**A**), or 24 h later (**B**). The protein profile was monitored continuously at a wavelength of 280 nm.

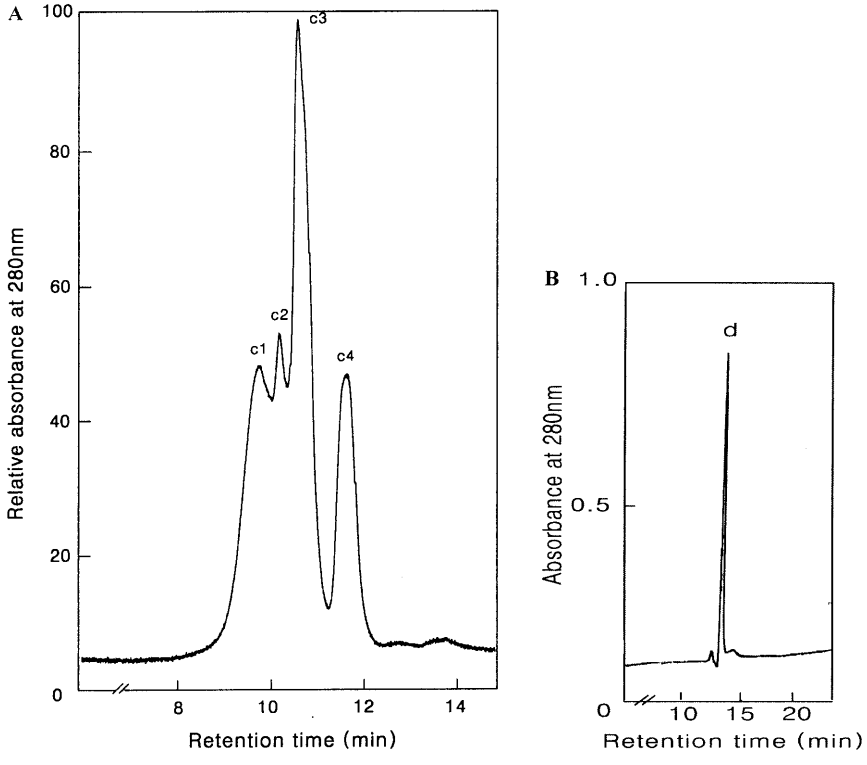

Fig. 4. Further purifications of fractions c, d in Fig. 2 by HPLC under 0.5 M NaCl. **A.** Twenty microliters of fraction c from the previous purification in Fig. 2 were dialyzed against 50 mM Tris-Cl, pH 7.3, containing 0.5 M NaCl, 2 mM DTT at 4°C for 1 h and loaded onto the TSK-250 gel filtration column and eluted at a flow rate of 1 ml/min. **B.** Fifty microliters of fraction d from the previous purification in Fig. 2 were loaded onto the TSK-250 gel filtration column under 50 mM Tris-Cl, pH 7.3, containing 0.15 M NaCl, 2 mM DTT and eluted at a flow rate of 1 ml/min. The protein profile was monitored continuously at wavelength of 280 nm.

Table 1. Elastase activity of each fraction from the TSK-250 gel filtration column chromatography in Fig. 4.

Fraction no.	Elastase activity (mM/mg/min)	
	SANA	Elastin
b	0	211
c 1	32	132
c 2	111	136
c 3	88	169
c 4	0	0
d	71	144

Fifty microliters of each fraction from the TSK-250 gel filtration column chromatography in Fig. 4 was incubated with SANA or elastin-Congo red in 50 mM Tris-Cl, pH 7.3, at 37°C. The elastase activities were measured spectrophotometrically at 410 or 492 nm by monitoring the concentrations of liberated *p*-nitroanilide or soluble elastin.

Table 2. Inhibitory effects of PMSF, DipF on SANA hydrolyzing activity.

Fraction no.	Residual elastase activity (% of control)	
	1 mM PMSF	20 μM DipF
c 1	8.5	29.5
c 2	1.6	21.9
c 3	1.8	25.8
d	25.5	35.76

Each fraction, which was purified by TSK-250 gel filtration column chromatography, was preincubated with 1 mM PMSF, 20 μM DipF in 50 mM Tris-Cl, pH 7.3, at 37°C for 20 min. The elastolytic activity with SANA hydrolysis was determined at 410 nm and was expressed as a percentage compared with the control activity in the absence of the inhibitor.

Table 3. Inhibitory effects of PMSF, DipF on the activity of elastin degradation.

Fraction no.	Residual elastase activity (% of control)	
	1 mM PMSF	20 μM DipF
b	86	79.7
c 1	13.2	0
c 2	5.8	0
d	0	0

Each fraction, which was purified by TSK-250 gel filtration column chromatography, was preincubated with 1 mM PMSF, 20 μM DipF in 50 mM Tris-Cl, pH 7.3, at 37°C for 20 min. The activity of elastin degradation was determined at 492 nm and was expressed as a percentage compared with the control activity in the absence of the inhibitor.

was weakly inhibited by PMSF, DipF compared to that of elastase. It was interesting to note that the reduced fraction c4 showed weak cathepsin G activity

Table 4. The comparison of NH_2-terminal amino acid sequences.

Fraction or comparable species		N-terminal sequences
Fraction	c 1	Ile-Val-Gly-Gly-Arg-Arg-Ala
	c 2	Ile-Val-Gly-Gly-Arg-Arg-Ala
	c 3	Ile-Val-Gly-Gly-Arg-Arg-Ala
	c 4	Ile-Val-Gly-Gly-**Ser**-Arg-Ala
	d	Ile-Val-Gly-Gly-Arg-Arg-Ala
Elastase[a]		Ile-Val-Gly-Gly-Arg-Arg-Ala
Cathepsin G[b]		Ile-**Ile**-Gly-Gly-Arg-**Glu-Ser**

[a] From Sinha *et al.*, 1987.
[b] From Salvesen *et al.*, 1987.

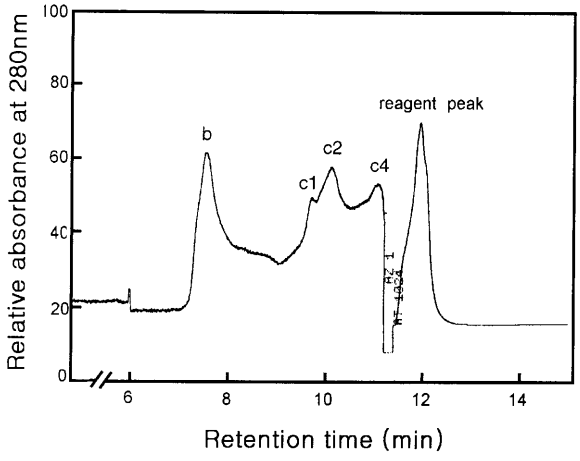

Fig. 5. HPLC analysis of a partially reduced fraction b. The fraction b from the TSK-250 gel filtration column chromatography (Fig. 2) in 100 mM Tris-Cl, pH 8.5, containing 6 M guanidine hydrochloride, was mixed with 10 μl of 2-mercaptoethanol. The mixture was flushed with nitrogen gas and then incubated at 37°C for 60 min. The reduced fraction b was analyzed on a TSK-250 gel filtration column in 50 mM Tris-Cl, pH 7.3, containing 0.5 M NaCl at a flow rate of 1 ml/min. The protein profile was monitored continuously at a wavelength of 280 nm.

(S.A. = 2.1 μM/mg per min). But the NH_2-terminal amino acid sequence of c4 was distinguished from those of elastase or cathepsin G. The residue that aligns with the Arg^5 of elastase is substituted with Ser^5 (Table 4).

Discussion

The five different molecular masses of HLElastases at approximately 72, 31, 29, 27, and 24 kDa could be purified effectively by three steps including Ultrogel AcA54 gel filtration, CM-Sephadex 25 ion exchange, and TSK-250 gel filtration chromatography. Three distinct polypeptide chains with molecular masses of

31, 29, and 27 kDa might be isoenzymes that have the same N-terminal amino acid sequences, Ile-Val-Gly-Gly-Arg-Arg-Ala, with their elastolytic activity conserved in the N-terminal structure. In contrast to Taylor's observations (Taylor and Crawford, 1975), the 24 kDa molecule which showed an identical NH_2-terminal amino acid sequence to the elastase (Table 4) might be a degraded fragment of native elastase. In our present study, proteolysis of HLE appears to be the cause of the lower molecular mass at 24 kDa (Fig. 3B). It was interesting to note that the autolytic product fully retained the activity of elastase on elastin dagradation or SANA hydrolyzation. Since the N-terminal amino acid sequence of the autolytic product was identical to the native elastase, the C-terminal amino acids of native elastase might well be cleaved by autolysis. The inhibitory effects of PMSF, DipF on elastolytic activity was similar to that of native elastase, suggesting that the N-terminal domain of neutrophil elastase plays an essential enzymatic role. We suspected that Val^{190}, or Ala^{187}, bonds might be the cleaving site during autolysis since the molecular mass of the autolytic fragment was 24 kDa and it preferentially cleave Val-X bonds and, to a lesser extent, Ala-X bonds (Blow, 1969). As a consequence, 29 or 32 amino acids in the C-terminal region, including the Cys^{169}-Cys^{194} sulfide bridge, might be deranged. However, the integrity of the amino acid in the active site was not disrupted since the autolytic fragment showed similar activity to that of the native form. In an attempt to ascertain the importance of disulfide bridging to the integrity of the molecule, we have used the known X-ray crystal structure of HLElastase to construct the active site of the enzyme (Bode *et al*., 1986; Sinha *et al*., 1987). Thus, the three intramolecular disulfide bridges in the N-terminal region, except the Cys^{169}-Cys^{194} bridge at the C-terminal site which was deranged during autolysis, might be important to support this conformational structure (Fig. 6).

The 72 kDa molecule might be a trimer formed between native enzyme molecules since the reduced 72 kDa molecule was separated into three peaks, c1, c2, and c4 (Fig. 5). When one or two of the native elastases formed a trimer with the c4 molecule which was eluted in peak c4 of Fig. 5, the elastolytic activity was increased 1.5 times higher than native elastase alone. This elastolytic activity was weakly inhibited by PMSF, DipF. Despite the c4 fraction not having elastolytic activity, it plays an important role in the potentiation of the elastolytic activity of native elastase. Several laboratories have reported that leukocyte elastase is inefficient in solubilizing elastin compared with pancreatic elastase (Boudier *et al*., 1984; Reilly *et al*., 1984). But Boudier *et al*. (1981) have shown that polymorphonuclear leukocytes contain about equal amounts of elastase and cathepsin G and that the elastolytic activity of

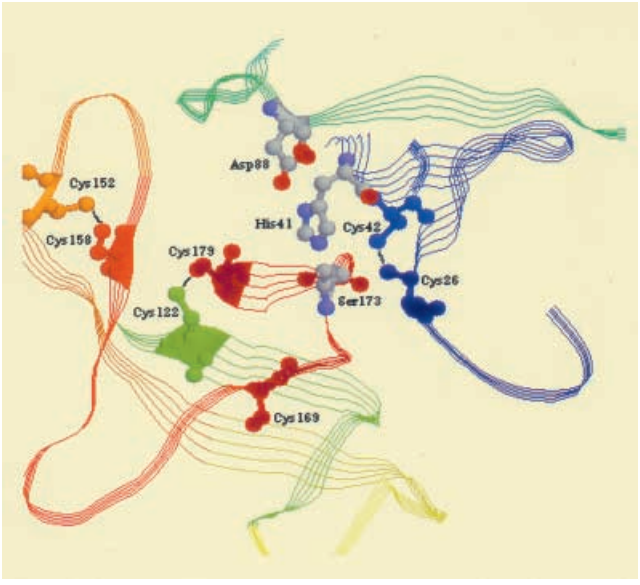

Fig. 6. Tertiary structure of HNElastase. Crystallographic data was obtained from the Brookhaven National Laboratory Protein Data Bank and the structure was drawn by the Molecular Visualization Program (Roger Sayle Biomolecular Structure Cloxo Research & Department, Greenfold, U.K.).

such a mixture is 5–6 times higher than that of elastase alone. In the present study, we also found that the potentiation of elastolytic activity could be a result of trimerization between native elastase and the c4 molecule. This enhanced elastolytic activity was negligibly regulated by serine protease inhibitors, such as PMSF, DipF. Although the c4 molecule showed weak cathepsin G activity, it has a different NH_2-terminal amino acid sequence which does not correspond to those of the azurophil granule enzymes, including elastase or cathepsin G. Therefore, we propose c4, showing a cathepsin G-like property, as a novel enzyme which has an important role in the pathological processes of neutrophil proteases in the chronic inflammatory diseases.

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